Influence of Dietary Arginine on Behavior

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Abstract

Autism is characterized by inappropriate social interactions, deficits in verbal communication and facial expressions, and ritualistic and repetitive behaviors. Recent evidence suggests the gaseous neurotransmitter nitric oxide (NO) may regulate certain behaviors characterized by autism-like symptoms. Inhibition of NO leads to hyper-aggression and excessive mounting which may represent inappropriate social interactions along with ritualistic and persistent behaviors observed in autistic-like symptoms. NO inhibition also decreases social investigation towards a social cue of a novel mouse which may reflect deficits in social communication. Many previous studies inhibited NO through deletion of the gene that codes for the synthetic enzyme or a drug induced inhibition of the synthetic enzyme, however, manipulation of dietary intake of the NO precursor, L-arginine, has not been extensively examined for regulation of these same behaviors. L-arginine is a common amino acid found in multiple foods. In the brain L-arginine is converted into NO by the enzyme neuronal nitric oxide synthase (nNOS). In this study, mice fed a diet consisting of a an L-arginine-depleted, L-arginine-supplemented, or control level of L-arginine underwent a series of tests designed to investigate social behaviors and affective responses. I hypothesized that a diet depleted of arginine would result in hyper-aggressive behaviors illustrated by persistent attacks on an intruder mouse along with a decrease of interactions with a socially-novel mouse. An arginine-deficient diet should also evoke persistent swimming in the Porsolt forced-swim test. Quantification of neurons positively stained for citrulline, a byproduct of NO synthesis, will indicate an overall decrease of nitric oxide activity in the brain of mice fed

an arginine-deficient diet. Conversely, a diet fortified with L-arginine will result in behaviors and hormonal levels opposite to those observed in mice fed an arginine depleted diet. If behavioral effects are congruent with autistic-like phenotypes, then investigation towards underlying mechanisms of autism through arginine depletion should be considered. This study may also provide a foundation for studying other disorders characterized by social withdrawal, hyper-aggression, or anxiety.

Introduction

Developing rodent models of neuropsychiatric disorders has a long and illustrious history. Although a rodent model cannot replicate a human disease, fundamental symptoms can be approximated for the purpose of testing hypotheses about the causes of abnormal human conditions. Hypotheses about atypical behavior and its underlying mechanisms can be tested using techniques that alter the biochemical or genetic make-up of a rodent. Specific behavioral testing, such as the elevated plus maze for anxiety-like responses or Porsolt's forced swim test for depressive-like responses, analyze behavioral changes that result from these altered molecular and physiological pathways. This provides useful information that facilitates the discovery of psychopharcological treatments for many mental illnesses and neurological diseases (Contarino, Heinrichs, & Gold, 1999; Crawley, 2004).

One disorder currently receiving a lot of attention is autism. Autism has been a particularly difficult disorder to study due to the wide variety and severity in symptoms. However behavioral abnormalities associated with this disease are usually characterized by three main symptoms: inappropriate social interactions (Lord, Cook, Leventhal, & Amaral, 2000; Volkmar & Pauls, 2003), deficits in verbal communication and facial expressions (Doussard-Roosevelt, Joe, Bazhenova, & Porges, 2003; Lord, Cook, Leventhal, & Amaral, 2000), and ritualistic, repetitive behaviors (Frith, Morton, & Leslie, 1991; Hollander, Phillips, & Yeh, 2003). Despite the unknown etiology of autism, many researchers are attempting to create animal models of behavioral abnormalities to further study the etiology of this disorder. One such model suggests altered levels of the gaseous

neurotransmitter nitric oxide (NO) may result in deficits of these same behavioral types (Zoroglu et al., 2003).

NO acts as a chemical messenger that has multiple functions including neuromodulation in the central and peripheral nervous systems, mediating the vasodilator tone that is essential for blood pressure regulation, and contributing cytotoxins of immune cells (Nelson, Kriegsfeld, Dawson, & Dawson, 1997) In the brain, NO is rapidly synthesized and acts as a gaseous neurotransmitter with a very short half-life of 2-5 seconds. Many studies have manipulated NO by affecting the synthetic enzyme, nitric oxide synthase (NOS). Three distinct NOS isoforms have been discovered: I) endothelial NOS (eNOS-3) in the tissue of blood vessels, II) inducible NOS (iNOS-2) found in macrophages, and III) nNOS-1 found in neurons (Nelson, Kriegsfeld, Dawson, & Dawson, 1997).

As a neurotransmitter, NO regulates social behaviors. In male mice, selective depletion of the nNOS gene (nNOS-/-) results in hyperaggressiveness. Mice that lack the gene to make nNOS continue attacks towards an intruder mouse despite obvious signs of submission. nNOS-/- mice also persist in mounting anestrous females despite vocal and behavioral protesting (Nelson et al., 1995). These persistent behavioral patterns despite submissive postures and vocal protesting may reflect deficits in key areas of the brain important for processing social information and responding accordingly. Further these behavioral patterns suggest deficits in changing established behaviors leading to repetition and persistency.

The current DSM-IV identifies many subtle deficits in social interaction as sufficient criteria for Autism (Diagnostic, 1994). Some of these behavioral abnormalities

include deficits in non-verbal expressions, deficits in social approach, and abnormal usage of language. In rodent models, studying social behaviors characteristic of humans are extremely difficult to study due to the complexity of construct (Moy et al., 2004). However, recent studies provide insight into the regulatory role NO has on social behaviors. Depletion of NO via pharmacological inhibition with the selective nNOS inhibitor 3-Bromo-7-Nitroindazole (3-Br-7NI) or via gene deletion reduces investigation time towards an unfamiliar mouse separated by a barrier in single-housed male mice (Trainor, Workman, Jessen, & Nelson, 2007). Exploration towards urine of an unknown mouse is a good marker for social investigation. Mice deposit pheromones in their urine to communicate specific information towards other mice, such as age, gender, and sexual receptivity. Therefore decreased investigation time towards another mouse's urine would indicate deficits in social interaction. Further, mice administered 3-Br-7-NI show reduced investigation time towards urine of an unknown mouse in both single-housed and pair-housed mice (Trainor, Workman, Jessen, & Nelson, 2007).

Reduced investigation cannot be attributed to deficient olfactory senses. Although NO inhibition decreased investigation time towards urine of an unfamiliar mouse in both single-housed mice and pair-housed mice, single-housed nNOS-/- mice
mice spend more time investigating compared with pair-housed WT mice. This demonstrates nNOS inhibition does not block the ability to recognize a social stimulus. Instead, nNOS inhibition appears to decrease motivation to explore a new social stimulus (Trainor, Workman, Jessen, & Nelson, 2007). Additionally latency to find a hidden cookie between nNOS-/- mice and WT mice does not differ (Nelson et al., 1995) further suggesting reduced investigation cannot be attributed to deficient olfactory senses.

Behavioral studies of mice using deletion of specific genes suffer the criticism that the gene product is not only missing from the testing period but throughout development when critical activation of compensatory mechanisms may be affected (Nelson, Kriegsfeld, Dawson, & Dawson, 1997). Further, beha(Boris & Mandel, 1994; Carter et al., 1993)vioral studies using pharmacological means to manipulate a gene or biochemical pathway requires surgery or daily injections not only runs the risk of severely injuring the animal but it also alter stress levels or immune responses which may alter subsequent behaviors. Manipulating dietary intake of nutrients and amino acids avoids these problems while retaining the ability to influence pathology and behavior.

Intake of nutrients and amino acids influences behavioral functioning along with psychological outcomes and provides therapeutic treatment options for certain neuropsychiatric disorders. Eliminating intake of preservatives and artificial food coloring in children with attention deficit hyperactive disorder (ADHD) increases response rate in retention tests (Boris & Mandel, 1994; Carter et al., 1993). Also tryptophan-depleted diet improves psychotic symptoms in schizophrenic patients, although minimally (Rosse et al., 1992; Sharma et al., 1997). Because L-arginine acts as a precursor to NO in the brain, it may provide therapeutic benefits to people experiencing deficits in social interactions or other autistic-like symptoms.

L-arginine is a non-essential amino acid that metabolizes with nNOS to create NO and the byproduct citrulline. An increase of L-arginine directly results in an increase of synthesized NO (Long, et. al. 2006). Many studies involving NO manipulation use mice lacking the nNOS gene (Nelson et al., 1995; Orlando, Langnaese, Schulz, Wolf, & Engelmann, 2008; Workman, Trainor, Finy, & Nelson, 2008). Other studies manipulate

NO levels with intraperitoneal injections of L-arginine (Guan, Yaster, Raja, & Tao, 2007; Khan, Tachibana, Hasebe, Masuda, & Ueda, 2007). However, NO manipulation through dietary intake of L-arginine has not been extensively studied despite the presence of arginine in many common foods, including chocolate, wheat, dietary products, and poultry (Cooper, 1997; Murray & Pizzorno, 1990).

Recent evidence suggests differing concentrations of dietary arginine affects specific behaviors. Pigs fed a fortified diet of L-lysine and L-arginine show decreased stress response during transportation (a stressful environment) (Srinongkote, Smriga, Nakagawa, & Toride, 2003). Mice fed an L-arginine depleted diet display less motor and exploratory activity and an increase of motor and exploratory activity than those fed an L-arginine rich diet (D'Hooge, Marescau, Qureshi, & De Deyn, 2000). Although behavior was influenced by dietary levels of L-arginine, specific behavioral testing will need to be conducted to elucidate specific behaviors that L-arginine affects.

I examined the influence of dietary L-arginine manipulation on social behaviors and affective responses to nNOS inhibition. I hypothesized that a diet depleted of arginine would result in hyper-aggressive behaviors illustrated by persistent attacks on an intruder mouse along with a decrease of interactions with a socially-novel mouse. An arginine-deficient diet should also evoke persistent swimming in the Porsolt forced-swim test. Quantification of neurons positively stained for citrulline, a byproduct of NO synthesis, will indicate an overall decrease of nitric oxide activity in the brain of mice fed an arginine-deficient diet. Conversely, a diet fortified with L-arginine will result in behaviors and hormonal levels opposite to those observed in mice fed an arginine depleted diet.

Methods

Animals

Thirty adult wild-type mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were 1 month old upon arrival and placed on a standard diet (Harlan Teklad Rodent Diet) for 5 days to allow for habituation to housing conditions. All mice were singly-housed in polypropylene cages (dimensions: 27.8 x 17.5 x 13 cm) and had *ad libitum* access to food (Harlan Teklad Rodent Diets South Easton, MA) and filtered tap water. The light cycle was 16L: 8D with the dark phase beginning at 1500 h Eastern Standard Time (EST). After 5 days mice were placed on an L-arginine-defined diet containing either 0.0%, 1.5%, or 5% L-arginine (Harlan Teklad TD.04391, TD.07826, and TD.07827).

Behavioral Testing

Behavioral testing was conducted 14 days after feeding began This is sufficient time to yield stable nitrate concentrations in urine (Srinongkote, Smriga, Nakagawa, & Toride, 2003). Before each test mice were allowed 15 minutes to acclimate to the testing rooms and all behavioral testing was conducted during the dark phase.

Open Field

The test chamber consisted of a 60 cm^3 clear Plexiglas box lined with corncob bedding. Movement was tracked by a series of infrared lights surrounding the box. Between tests, the chamber was cleaned using 70% ethanol (EtOH). Each test session was 30 mins in duration. Total locomotor activity, percent time spent in the center of the

open field, and rearing were quantified by the PAS software package (San Diego Instruments, San Diego, CA).

Elevated Plus Maze

The maze was ~ 1 M above the floor and consisted of two open arms bisected by two enclosed arms with dark-tinted acrylic. Mice were placed in the center of the maze, facing a closed arm and were recorded from a camera suspended from the ceiling for 5 mins. The maze was cleaned with 70% EtOH between tests. An arm entry was scored when two forepaws entered an open arm. Latency to enter an open arm, total time spent in the open arms, and number of open armed entries was scored and analyzed using Observer software (Noldus Corp., Leesburg, VA).

Sociability/Novel Test

The testing chamber was 20 cm x 40.5 cm x 22 cm. Dividing walls were made from clear Plexiglas, with small openings allowing access into each chamber. In the first 5 mins, mice were allowed to acclimate to the testing apparatus which consists of a large rectangular chamber divided into 3 areas. The doorways leading to the two side chambers were left on to obstruct entry. During the next 10 mins, an unfamiliar stimulus mouse (S1) was placed into one of the chambers underneath a cup with holes allowing for olfactory and visual stimulation to take place and the doors were removed. The cup prevented any agonistic encounters while allowing transmission of visual and olfactory cues. During the second 10 min session, a second stimulus mouse (S2) was placed into the opposite side chamber. Test mice had the option of investigating the unfamiliar mouse or the already-

investigated mouse. All protocols followed (Nadler et al., 2004). The stimulus mice were the same age and genotype as the test mice and were acclimated to the cup for a 30 min period a day before testing. The location of the first stranger mouse alternated between left and right chambers between trials to control for any preference for a particular side of the chamber. Behavior was recorded on video under a dim red light. The chambers were cleaned with soap and water and the cups were cleaned using 70% EtOH between each test. Latency to enter each side chamber, time spent in each side chamber, and time spent actively investigating the mice were recorded. Active investigations were defined as sniffing while in close proximity to stimulus mice.

Forced Swim Test

Mice were placed into a cylindrical tank filled with water, with a temperature ranging from 23°C-28°C, for a 10 min testing period. Testing was recorded and scored based on duration of floating, latency to float, and total number of floats. Floating was described as complete limb immobility or movement only necessary to keep the head afloat. Water was changed after each test. (Porsolt, Anton, Blavet, & Jalfre, 1978)

Re*sident-Intruder Test*

Each mouse was confronted by an unknown mouse for a 7 min period. Intruder mice were matched to resident mice based on weight. Due to availability, the mean weight of the intruder mice was .81g heavier than resident mice. Intruder mice were marked on their tail for identification. No wounds were observed in any test. Aggressive behaviors were scored as following: tail rattling, biting, and boxing (Nelson et al., 1995).

Perfusions

Mice were anesthetized with isoflurane and injected with sodium pentobarbital before perfusion through the left ventricle with ice cold phosphate buffered saline (PBS) followed by a fixative compound of 3% paraformaldyde and 1% gluteraldehyde in PBS. Brains were removed and postfixed over-night then transferred to 30% sucrose in phosphate buffer until they sunk to the bottom. Then brains were frozen using methylbutane and dry ice. They were stored at -80º C until slicing.

Statistical Analysis

Data for behavioral tests were analyzed using one-way ANOVAs, testing for the effects of arginine concentration on specific behaviors. In the elevated plus maze data, two mice were removed from analysis, both from the supplemented group, due to error in maze configuration. In the sociability/social novelty test four mice were removed from analysis, two from the supplement group, one from the control group, and one from the depleted group, due to error in chamber configuration and tape malfunction. In the resident-intruder test, one mouse was taken out of the control group due to a large weight differential between resident and intruder. ANOVAs were considered statistically significant if $p < 0.05$.

Results

There was an error in the diet, provided by the manufacturer, resulting in the arginine-depleted group to ingest approximately 1.7% arginine in their daily diet, making all results based on depleted arginine in a diet null and void. However, conclusions can still be drawn with respect to enriched levels of arginine in a diet.

Mice fed a depleted diet of arginine had more floating bouts than mice fed a supplement diet ($F_{2,26} = .857$, $p < 0.05$) (Figure 1). However, diet did not affect latency to float or total duration floating (Figure 2).

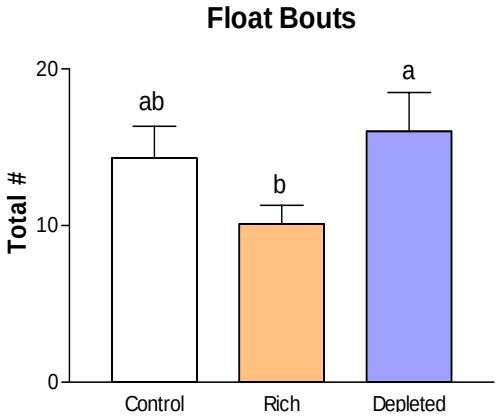

Figure 1. The depleted group experienced more floating bouts than the rich group. $F_{2,26} = .857$, $p < 0.05$.

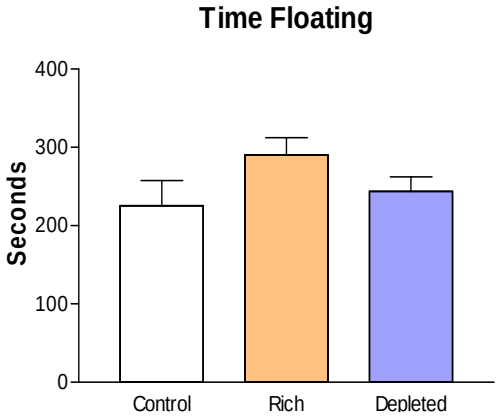

Figure 2. Total time spend floating did not differentiate between each group.

There also was a difference between time spent actively investigating S2 between the arginine-depleted and rich groups (Figure 3B) ($F_{2,23}=5.136$, $p < 0.05$). However diet did not affect transitions between chambers, duration in each chamber, total number of social encounters, latency to enter each chamber, or latency to investigate an unknown mouse with either S1 or both S1 and S2 housed in separate chambers.

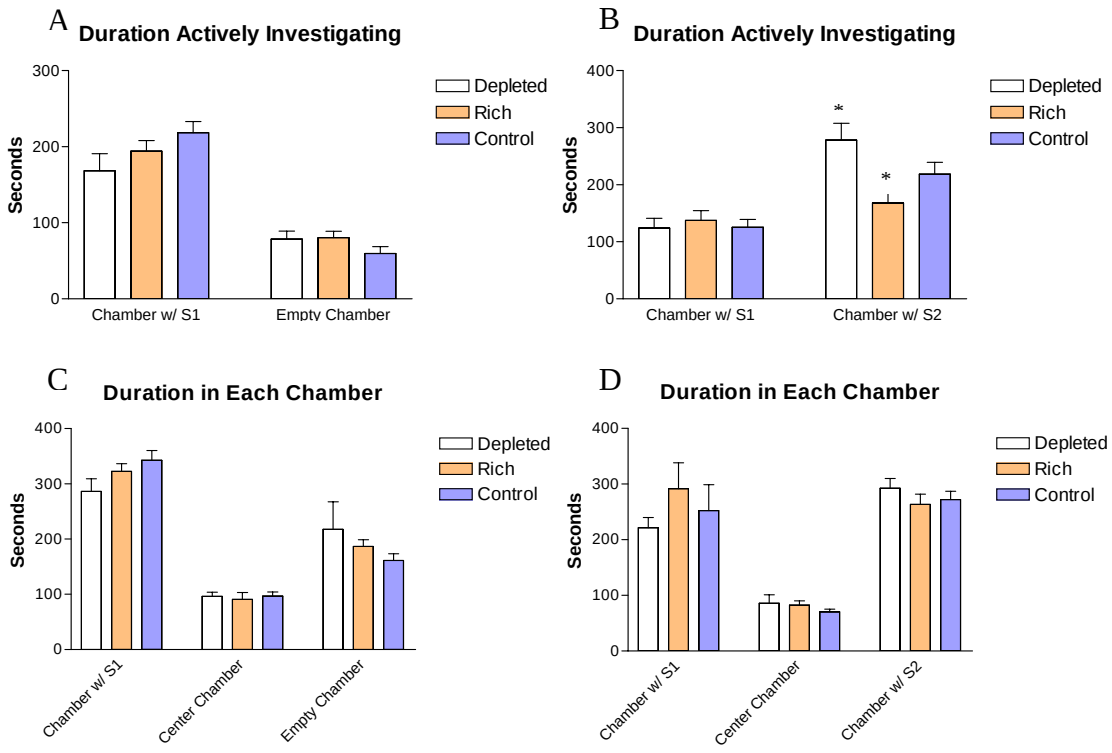

Figure 3. A) There were no differences between time spent actively investigating S1. B) The depleted group spent more time investigating S2 compared to the rich group ($F_{2,23}$=5.136, $p < 0.05$). C-D) Time spent in each chamber did not differ between groups with only S1 or with S1 and S2.

Diet did not alter total locomotor activity and tendency to explore the center of the open field (Figure 4). Diet also had no significant effect on open arm entries, latency to enter open arms, and duration in open arms between each group (Figure 5). There was no effect in total amounts of biting, tail rattling, or boxing between each group. However the control group had an elevated mean for both biting (M= 18.000) and tail rattling (M=2.778) although nonsignificant (Figure 6 and 7).

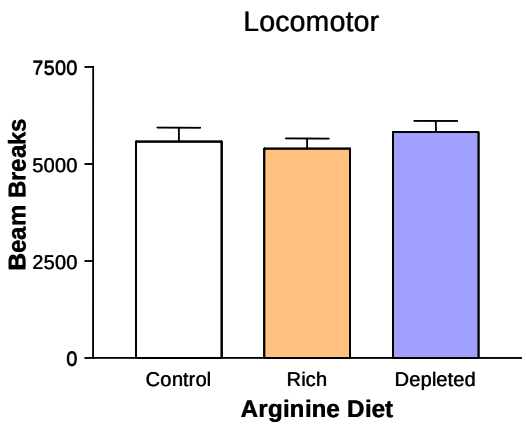

Figure 4. Total locomotor activity in the open field expressed by total beams broken throughout the 30 min test.

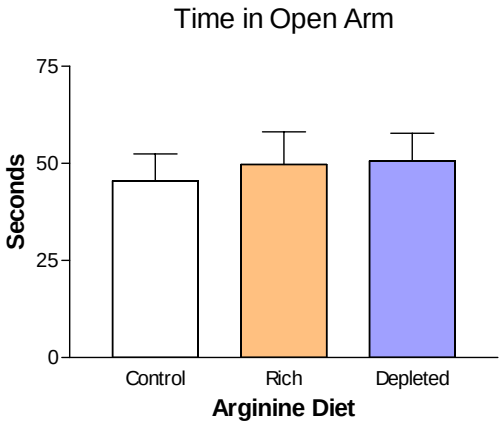

Figure 5. Total time spend in the open arms of the elevated plus maze. No significant results were found.

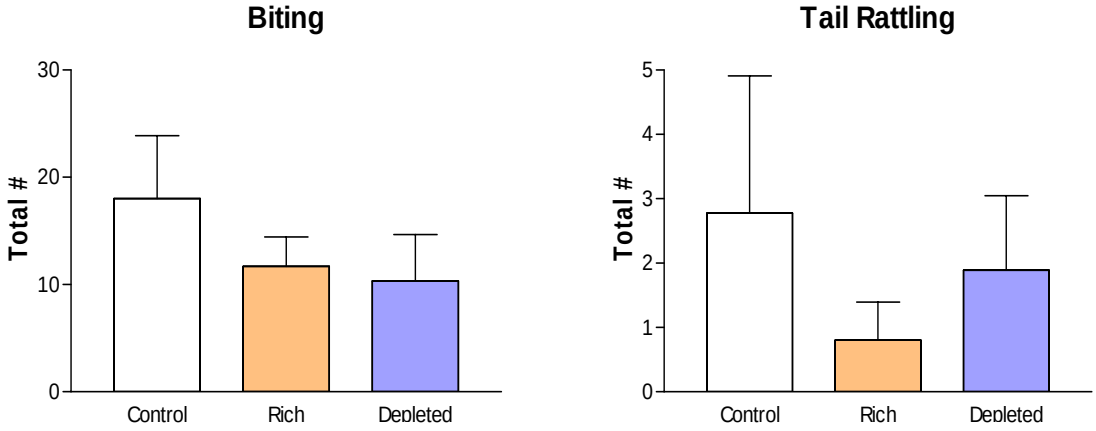

Figure 6. Although nonsignificant, there was a trend for the control group to show more aggressive behavior (biting and tail rattling) towards the intruder mouse.

Discussion

Although there was a difference in total number of floating bouts between the rich and depleted group, total time spend floating and latency to float did not differ. Taken together, along with the fact the depleted group ingested arginine, an unknown variable must have accounted for the difference. The same can be said for the difference in S2 investigation between the rich and depleted group. Total time spend in each chamber along with latency to enter each chamber and total number of S2 investigations did not differ suggesting diet did not cause the significant difference between the groups.

These data indicate an enriched amount of arginine in a diet does not alter anxiety-like behaviors in either the open field test or the elevated plus maze. It also does not alter social investigation of an unknown mouse or influence preference for a novel mouse over an already investigated mouse. A supplemented amount of arginine also does not alter depressive-like responses or aggression in the resident-intruder test. However, in the resident-intruder test, there was a trend for the control group to display more biting (M=18.00) and Tail Rattling (2.778). This suggests an inhibitory role on aggressive behavior with the presence of arginine in a diet or, vice versa, an increase of aggressive behavior with a lower level of arginine in a diet.

Past research indicates nNOS inhibition is anxiogenic (Spiacci, Kanamaru, Guimaraes, & Oliveira, 2008; Srinongkote, Smriga, Nakagawa, & Toride, 2003; Workman, Trainor, Finy, & Nelson, 2008); mice typically decrease open arm exploration in the elevated plus maze. This also increases staining for corticotropin-releasing hormone (CRH) (Workman, Trainor, Finy, & Nelson, 2008). CRH induces adrenocorticotropic hormone (ACTH) release from the pituitary which stimulates coritsol

(or corticosterone), the major stress hormone, release from the adrenal cortex (Owens & Nemeroff, 1991). Nitric oxide is colocalized with CRH within the paraventricular nucleus (Yamada, Emson, & Hokfelt, 1996) further indicating NO can modulate the effects of CRH on exploratory behavior. It remains unclear if arginine depletion can influence anxiogenic behaviors.

NO inhibition appears to be involved with anti-depressant behaviors. NO inhibition results in persistent swimming in the forced swim test and longer suspension in the tail suspension test. Acute administration of NOS inhibitor, N^G-nitro-L-arginine (L-NA), decreases immobility in the forced swim test. This effect was reversed by arginine indicating that NO is involved with antidepressant-like behaviors (Karolewicz, Paul, & Antkiewicz-Michaluk, 2001). Low doses (25 nmol) of L-NAME, a non-specific NOS inhibitor, also decease immobility time and latency to swim in the forced swim test. These antidepressant effects are reversed when higher doses (200 nmol) of L-NAME are administered (Spiacci, Kanamaru, Guimaraes, & Oliveira, 2008). Together, these results suggest NO has a complex regulatory role on depressive-like behaviors and how arginine will influence this behavior remains unknown.

Recent behavioral tests have been developed to quantify appropriate social interaction in mice. The three chambered sociability and social novelty preference task allows for accurate quantification of social behaviors in rodents and offers construct validity towards social deficits in numerous neuropsychiatric disorders such as autism (Nadler et al., 2004). Evidence suggests inhibition of NO decreases social investigation towards other mice. nNOS-/- decrease investigation time towards urine of an unknown mouse. Pharmacological inhibition of NO also results in decreased interaction time

towards novel juvenile mice (Black, Simmonds, Senyah, & Wettstein, 2002) an abnormal characteristic of social rodent species. Based on these studies I would expect arginine depletion would decrease investigation towards the first stranger mouse. Because social deficits in autism may appear as inappropriate or indiscriminate approaches to strangers, rather than an overall lack of social approach, I would also expect arginine-depleted mice to decrease investigation time towards a socially-novel mouse compared with a familiar mouse.

Although nonsignificant, there was a trend for the control group to be the most aggressive of the three groups of mice. Considering the control groups were fed the least amount of arginine (1.5%) this may indicate arginine depletion results in increased aggression and arginine supplementation may decrease aggressive behaviors. Studies of inter-male aggression in an intruder-resident model (Nelson et al., 1995) revealed nNOS-/- mice display excessive aggression towards intruders compare with WT mice. Interestingly, latency for first attack did not differ between these groups. The difference between groups came after the initial attack. nNOS-/- mice displayed 3-4 times the aggressive encounters compared to WT mice with most of these attacks being initiated by the nNOS-/- residents. This increased aggression after first attack suggests nNOS-/- mice are unable to recognize the submissive posture displayed by the intruder and continue their attacks despite obvious surrender. Depletion of arginine should result in similar behaviors. I would expect an increase in aggressive behaviors (i.e. tail rattling, boxing, and biting) in the resident-intruder test.

Because the arginine-depleted group was fed a diet consisting of arginine, most results were null and void. Further testing needs to be conducted to examine the influence

arginine has on specific behaviors, i.e. anxiety-like, depressive-like, social investigation, and aggression.

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